Potential clinical error arising from use of HbA1c in diabetes: effects of the Glycation Gap

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ABSTRACT

The Glycation Gap (GGap) and the similar Hemoglobin Glycation Index (HGI) define consistent differences between glycated hemoglobin and actual glycemia derived from fructosamine or mean blood glucose respectively. Such a disparity may be found in a substantial proportion of people with diabetes, being > than 1 unit of glycated HbA_{1c}% or 7.2 mmol/mol in almost 40% of estimations. In this review we define these indices, explain how they can be calculated and that they are not spurious, being consistent in individuals over time.

We evaluate the evidence that GGap and HGI are associated with variation in risk of complications and mortality and demonstrate the potential for clinical error in the unquestioning use of HbA_{1c}. We explore the underlying etiology of the variation of HbA_{1c} from mean glucose in blood plasma including the potential role of enzymatic deglycation of hemoglobin by fructosamine-3-kinase. We conclude that measurement of GGap and HGI are important to diabetes clinicians and their patients in individualization of therapy and the avoidance of harm arising from consequent inappropriate assessment of glycaemia and use of therapies.

I. INTRODUCTION

HbA_{1c} has become the *sine qua non* of diagnosis and of clinical study outcome measures with few diabetes professionals questioning its apparent validity (1). Nevertheless a historical perspective shows that, in fact, many doubts have been aired over the last 30 years about such an unquestioning assumption (as recently underlined by Cohen and colleagues (2)). Whilst for the majority of patients with (or suspected of having) diabetes mellitus the use of HbA_{1c} provides a tool that yields helpful guidance in diagnosis and treatment, there is an increasing body of evidence that for a substantial minority a more nuanced and individualised approach

is appropriate (3). In short, for these patients the blunt use of HbA_{1c} to guide treatment and diagnosis may lead to significant clinical errors. It is therefore important for those involved in the care of patients to understand the impact of the "Glycation Gap" (GGap) and its sister the "Hemoglobin Glycation Index" (HGI) on the validity of HbA_{1c} measurements (4–6).

II. Non-enzymatic glycation of blood proteins and use in estimating average glycemia

Hyperglycaemia of diabetes is associated with increased glycation of free amino-groups in proteins. Protein glycation is a key factor leading to vascular complications and furthermore, when occurring in erythrocyte proteins, provides the widely used index of average glycaemia, HbA_{1c} or glycohaemoglobin (GHb) (7). Glycation occurs as a result of the well-known reaction of carbohydrate moieties with amino groups of proteins known since 1910 as the Maillard, or more specifically when involving glucose, as the Schiff reaction. The aldimine product of the Schiff reaction undergoes slow but reversible rearrangement to the Amadori ketoamine product. The ketoamine is then slowly converted to advanced glycation end-products which comprise a wide range of chemical moieties which contribute to the development of complications of diabetes (for review see Zhang et al., 2009 (8))

Accurate quantification of glycaemia with reliable and practicable tests was historically a challenge. Before the development of accurate point-of-care devices blood glucose measurement and monitoring depended on inaccurate clinic or home based "stick" testing of blood glucose or else necessarily infrequent laboratory measurements.

As early as 1964, an unusual abnormal hemoglobin, HbA_{1c} ("blocked" at the N-terminus of the β-chain), was identified chromatographically (9). By the mid 1970's this was shown to be

a “glycosylated” variant which was elevated by approximately 2 fold in patients with diabetes along with other variants HbA_{1a} and HbA_{1b} compared to those without (10,11). HbA_{1c} was later shown to be the majority sub-component of total glycated hemoglobin, resulting from non-enzymatic glycation at the N-terminal valine of the beta chain of hemoglobin A; other glycations by glycolytic intermediates fructose-1,6-bisphosphate and glucose-6-phosphate produce variants such as HbA_{1a} and Hb_{1b} and glycation at amino groups of intra-chain lysines also occur but do not affect the chromatographic mobility of Hb. Originally referred to as glycosylated hemoglobin, the concept of glycation, a non-enzymatic reaction between glucose and free amino-groups on proteins, was developed to distinguish this process from the post-translational glycosylation of proteins and the use of the name “glycated hemoglobin” was proposed in 1983 (IUPAC) (12). As described above, glycation results from initial reversible reactions - the Schiff/Maillard reaction producing an aldimine followed by a further Amadori rearrangement to a more stable glycated ketoamine (proteins thus containing fructosyl-lysine or fructosyl-(N-terminal) amino acids). Subsequently clinical studies confirmed that HbA_{1c} could be used as a measure of glycaemic control (13), its assays are now standardised (14), and currently glycated HbA_{1c} is considered the gold standard measure of glycaemia over the preceding 3 months, closely associated with key microvascular complications in diabetes, proven risk reduction in complications with improvement in HbA_{1c} towards the normal non-diabetic range and more recently implemented internationally in the diagnosis of diabetes mellitus (1, 15,16).

However, a similar non-enzymatic glycation process occurs extracellularly with plasma proteins, predominantly albumin. Fructosamine is a marker derived from all ketoamine products occurring as a result of glycation of serum proteins and is measured by the nitroblue tetrazolium (NBT) assay. Fructosamine has been proven to be as reliable an indicator of

glycaemic control as HbA_{1c}, representing glycaemia over a shorter duration (because of the shorter half-life of serum proteins) than reflected by HbA_{1c} and associated with microvascular complications in diabetes similar to HbA_{1c} (17,18). Fructosamine as a measure of glycaemic control has been validated against glycated HbA_{1c} and blood glucose (19,20). Newer assays for fructosamine estimation, are based on more specific enzymatic ketoamine oxidation compared to NBT reduction (which is subject to interference by endogenous reducing substances etc.), although the two assays correlate closely (21). Direct assay of glycated albumin, reflecting glycaemic control over weeks, has been used in some countries, has gained importance in glycaemic monitoring, and is associated with microvascular complications in diabetes (17, 22)

III. Limitations to the clinical utility of HbA_{1c} – consequences of GGap and HGI

Current strategies in the management of glycemia in diabetes rely heavily on HbA_{1c}. Despite standardization of assays, discrepancy between HbA_{1c} and other assessments of glycemia is well reported and may affect accurate interpretation of glycemic control and its management (3–6, 23, 24). A variety of erythrocytic factors that impacts on red cell life span or turnover and glucose gradient across the red cell membrane, are known to affect HbA_{1c} independently of glycemia (25,26). Recent changes in glycemic control are possibly over-represented in HbA_{1c} i.e. HbA_{1c} does not reflect blood glucose levels equally over the previous 120 days. HbA_{1c} represents the net effect of several mechanisms, which may shift its direct glycation relationship with overall levels of glycemia. Various studies have calculated the deviation of HbA_{1c} co-utilising either fructosamine or blood glucose data referred to as the "Glycation Gap" (GGap, deviation of glycated HbA_{1c} from serum Fructosamine) or the Hemoglobin Glycation Index (HGI, discrepancy between HbA_{1c} and a predicted HbA_{1c} from date matched

mean blood glucose (MBG) estimations) (4–6, 27-29). HbA_{1c} could systematically deviate from glycemia as a result of elements that influence glycation within the red blood cells such that the HbA_{1c} might be lower (a negative GGap or low HGI implies a lower net rate of glycation) or higher (positive GGap or higher HGI, implying a higher rate of net glycation) than might be expected.

IV. Definition and calculation of GGap and HGI

Cohen *et al*. calculated the GGap as the difference between measured HbA_{1c} and the HbA_{1c} predicted from Fructosamine based on the population regression of HbA_{1c} on Fructosamine (5). Hempe *et al*. calculated the HGI as the difference between the measured HbA_{1c} and the predicted HbA_{1c} derived from date-matched mean blood glucose estimations (MBG) by regression (6). Similar methodologies were used by others whereby the GGap was calculated based on the regression of HbA_{1c} (y) versus Fructosamine (x) and HGI was calculated based on regression of HbA_{1c} (y) vs. MBG (x) (27-29). Statistically, the calculated GGap (or HGI) would thus be a linear function of HbA_{1c} and fructosamine (or MBG); GGap or HGI thus calculated would be significantly correlated with HbA_{1c} and hence it would be difficult to dissect the association with complications independent of HbA_{1c}. Furthermore the fructosamine-derived HbA_{1c} in GGap, or MBG-derived HbA_{1c} in HGI, would not be independent of HbA_{1c} – hence it would be statistically spurious to include HbA_{1c} in an analysis along with GGap or HGI (30).

We, in a previous study to assess the clinical impact of variability in HbA_{1c}, calculated the predicted HbA_{1c} from fructosamine by initially converting the fructosamine value into its standard normal deviate (SND) and then the fructosamine SND was converted to HbA1c equivalents ($FHbA_{1c}$) (3,4).

$$SND[f] = (\text{fructosamine} - \text{mean fructosamine}) / \text{SD fructosamine}$$

$$F\ HbA_{1c} = (SND[f] \times SD\ HbA_{1c}) + \text{mean } HbA_{1c}$$

The glycation gap was thus calculated as the difference between the true HbA_{1c} and the fructosamine derived standardised predicted $FHbA_{1c}$ (GGap = HbA_{1c} – $FHbA_{1c}$). In this methodology the $FHbA_{1c}$ is not derived from HbA_{1c} by correlation / regression methods (3,4). The normalized standard deviate re-allocation of fructosamine levels yields fructosamine based HbA_{1c} equivalent results with the same distribution, mean and standard deviation as HbA_{1c} without altering the rank position of fructosamine derived values.

Clinically, the analysis used in our published studies (3,4) can be used to obtain a simpler estimate of the GGap: Simultaneous HbA1c and fructosamine estimations can be utilised to calculate the predicted HbA_{1c} from fructosamine [$FHbA_{1c}$ = (((Fructosamine - 308/77) × 1.7) + 8.3] and the GGap (DCCT HbA_{1c} unit) is then calculated as the difference between HbA_{1c} (DCCT) and $FHbA_{1c}$

Recently others have used glycated albumin rather than fructosamine to estimate the GGap although this has not been validated against the established method described above (31). Although fasting blood glucose estimations are correlated well with mean glucose it is with a wide variance, and methodologies using 6 or 8-point glucose profiles provide better representation of mean glucose, and hence a relatively better metric to be utilized in the HGI calculation. Availability of continuous glucose monitoring (CGM) with high density of data and better reflection of postprandial peaks could help in mean glucose calculations (and most recently it has been suggested that CGM could be used to titrate HbA_{1c} and thus the GGap for

individual patients (2)), however this has not yet been explored in HGI calculation; it may be noted nevertheless that mean glucose profiles were similar comparing CGM and 8-point blood glucose testing (32).

Despite the availability of such new technologies in some arenas it should be recognized that CGM is yet to be widely available in a large proportion of clinical situations and that HbA1c and/or GA/fructosamine will continue to be the major methods of monitoring glycemia worldwide for many years. Crucially, the calculation of GGap and HGI will continue to provide important information in relation to individual risk of diabetic complications as discussed in section VIII below.

V. Alternative explanations of the GGap/HGI

The use of HbA_{1c} depends on the assumption that erythrocyte (intracellular) glucose concentrations are an accurate reflection of plasma (extracellular) glucose on the basis that erythrocytes express the constitutive glucose transporter GLUT1, however this assumption may be incorrect for a number of reasons and furthermore the utility of glycated hemoglobin as an indicator of average glucose over the half-life of hemoglobin of 3 months assumes that there is no further change in the glycated product and indeed that the half-life (or life span determined as the "mean RBC age", M_{RBC}) of erythrocytes is consistent between individuals which it demonstrably is not (2); Malka *et al.* recently suggested a mathematical model for calculating individual M_{RBC}, which measure they propose as the explanation for all individual non-glycemic Hb_{A1c} variability and as a correction to be used in patient-personalized assessment of Hb_{A1c} results (33). GGap and HGI could potentially be explained by genetic and racial differences in M_{RBC} as pointed out by Cohen *et al.* (2), in addition to other non-

glycemic factors including alterations in GLUT1 expression or activity or intracellular enzymatic deglycation pathways, which are discussed below (sections VIII and IX).

VI. Consistency of GGap and HGI

It has been hypothesised that GGap and HGI represent a spurious statistical phenomenon (30; 34) arising from regression analysis used in some methodologies and on this basis Lachin *et al.* (34) suggest that HGI is not completely glycemia-independent and hence not an independent predictor of complications (see section VII). Our method of calculating GGap using the standardized normal deviate (section IV) avoids this problem (3,4), furthermore the consistency of GGap and HGI mitigates against this criticism; hence GGap and HGI have both been shown to be consistent in individuals over time, indicating a constant variation in intracellular glycation compared to extracellular glycation or glycaemia as measured by serum Fructosamine or MBG (4–6). In a retrospective study on 2,263 individuals with diabetes, by using multiple simultaneously measured HbA_{1c} and fructosamine in the same individuals over an extended time period, we confirmed the GGap can be of substantial magnitude, that there is no significant within-subject variability in the GGap and that the direction of the GGap is consistent despite significant changes in HbA_{1c} and fructosamine over the time period (4) (these findings are updated, extended and explored in more detail below section X and Figs 1-3). Others have demonstrated such reproducibility of GGap: Cohen *et al.* reported the reproducibility of the GGap in 65-paired HbA_{1c}-fructosamine estimations separated by 23 weeks in a population with diabetes (5). In a population not known to have diabetes, Yudkin *et al*. (35) and Gould *et al*. (36) showed that the discrepancy between the HbA_{1c} relative to fasting and 2-hour blood glucose levels in the oral glucose tolerance test (OGTT) remained consistent over a 4.4-year period (29,30) . Similarly consistency in the HGI has been studied in 128 children with type 1 diabetes, and it was noted

to be consistent over a 2-year study period (6). It was shown that individuals consistently had the same direction and magnitude of HGI from repeated measurements of HbA_{1c} and MBG over a 2-year period in a clinic population of children and adolescents with type 1 diabetes.

A comparison of GGap and the HGI in 62 patients with type 1 diabetes confirmed that the two indices are highly correlated and consistent (37). In a study in monozygotic twins, GGap was suggested to be 69% inheritable, indicating the possibility of a genetic basis for the GGap (38).

Recent studies examining GGap and HGI in Korean patients with type 2 diabetes, in which glycated albumin rather than fructosamine was used in the GGap calculation, have confirmed the correlation between these the two indices and also further demonstrated their consistency, and interestingly that patients with a high HGI/positive GGap had a higher incidence of insulin use, albeit in a small study group (39). Akatsuka *et al.* suggest that the ratio of glycated albumin to HbA_{1c} in IFCC units is an accurate measure of GGap and might be useful as a reference for predicting risk of complications in type 1 diabetic children (40).

VII. Association of diabetes complications with GGap and HGI

GGap/HGI might alter an individual's risk of vascular complications for any given level of long-term glycaemic control by modifying one of the key pathologic processes, namely, protein glycation and the formation of advanced glycation end products. Hypothesizing that the GGap is a trivial nonsystematic event, unconnected to diabetes outcomes, it would not then be expected to be associated with distinct subpopulations of human diabetes or to have any sequelae in clinical outcomes. We have reported the direct associations between positive GGap and microvascular and macrovascular complications of diabetes that are logically

consistent with the glycation mechanism for complications (41). Belonging to the consistently positive GGap group was significantly associated with worsening retinopathy (odds ratio [OR] 1.96 [95% CI 1.31–2.9], P=0.001), increasing urine albumin Creatinine ratio (1.85 [1.14–3.01], P = 0.012), and the presence of established macrovascular disease (1.91 [1.18–3.09], P=0.008). Others have reported a similar relationship between the GGap/ HGI and retinopathy and nephropathy. Cohen *et al.* suggested that the GGap increased the risk of more advanced nephropathy 2.9-fold (5). Rodrıguez-Segade *et al.* examined 2,314 patients with type 2 diabetes for a mean of 6.5 years, dividing the cohort into tertiles based on the average of all individual GGaps, and showed that the mean GGap predicts the progression of nephropathy (42). In a study analyzing the data from DCCT, HGI was shown to be a significant predictor of retinopathy and nephropathy (43). Furthermore the HGI sub-group analysis of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial revealed that intensive treatment significantly reduced the primary composite outcome (first occurrence of non-fatal myocardial infarction, non-fatal stroke, or death from other cardiovascular causes) by 25% in the low HGI subgroup and by 23% in the moderate HGI subgroup but not in the high HGI sub-group where the primary outcomes were similar between the standard and intensive glycaemia treatment groups (44). In the ACCORD cohort, fasting glucose values were used to calculate the predicted HbA_{1c} and the HGI, potentially not taking into consideration of the effect of the post-prandial glucose variations that may have impacted on the HGI.

We also examined mortality in our cross-sectional, retrospective study and found that the adjusted all-cause mortality was higher (2-fold) both in the negative and positive GGap groups compared to neutral GGap cohort (41). Cid Alvarez *et al.* in a prospective cohort study of individuals with diabetes and without diabetes, with acute coronary syndrome,

demonstrated an association of increased mortality with higher GGap values (diabetes cohort HR IC 95%: 1.31 (1.14-1.50), P<0.001; and non-diabetic patients HR IC 95%: 1.30 (1.04-1.64), P=0.018) (45). HGI subgroup analysis in the cohort from ACCORD trial also suggested increased total mortality by 41% (P = 0.02) in the high HGI group but not in low and moderate HGI group, in those in the intensive treatment arm (44).

In the cohort of patients with type 2 diabetes studied in the Action in Diabetes and Vascular Disease (ADVANCE) Trial, HGI was found to be a strong predictor of microvascular, macrovascular complications and mortality irrespective of the treatment allocation (intensive vs. standard treatment) but no better than HbA_{1c}. Further it was noted that intensive control reduced mortality in the high HGI cohort (high measured HbA_{1c} relative to that predicted from MBG) (46). This result was inconsistent with the findings of the ACCORD trial however the difference in the treatment regimen in the 2 trials is likely to have contributed to the different findings.

Similarly to our findings of the relationship of GGap with mortality (41), in a study of 976 individuals with diabetes with ischaemic stroke, high and low HGI were linked to poor outcome, with a U-shaped association of HGI with prognosis being demonstrated (47).
Table 1 summarizes the published studies demonstrating the association between GGap/HGI and diabetic complications.

VIII. Possible contributions to the GGap / HGI

Since the GGap or HGI is a measure of the net difference between HbA_{1c} and Fructosamine or MBG, factors that affect either of these parameters could influence the GGap or HGI.

As previously indicated the time frame of glycemic attainment represented by fructosamine is shorter than that of HbA_{1c}, the glycation it indicates may be influenced by protein turnover rates and protein loss as proteinuria, and these factors may play a part in the GGap. Many have shown a good relationship between HbA_{1c} and fructosamine (3-5, 27). Fructosamine is known to be well associated with preceding blood glucose levels (19). A concern relating to a possibly confounding association of fructosamine levels with proteinuria has been raised, however in our study utilising regression analysis of fructosamine with multiple relevant clinical and biochemical factors we showed that, overall, they explained no more than 20% of the variance in fructosamine, which is to say 80% of fructosamine is not associated with any known influencing factor (41), amongst which urine albumin-creatinine ratio had the statistically weakest independent association, with an r^2 of 0.002 thus representing only 0.2% of the accountable variance of fructosamine.

A variety of factors independent of prevailing glycemia influence glycated HbA_{1c}. Genetic variations could influence HbA_{1c} through non-glycemic pathways and contribute to HbA_{1c}-glycemia discordance (54). A previous study has confirmed that glycation gap may be partly genetically determined and account for one third of the heritability of HbA_{1c} (38).

Bergenstal *et al*. demonstrated racial difference in the relationship between HbA_{1c} and glycaemia, confirming that HbA_{1c} levels overestimate the mean glucose concentration in black persons compared with white persons, suggesting that there may be racial differences in the glycation of hemoglobin (55). Such ethnic differences which have a likely genetic component have also been demonstrated in a new study by Hivert *et al.* (56) and the authors point out that an association of a genetic variant in *G6PD*, which is common in black ethnicities, is a factor identified in GWAS as a genetic determinant of Hb_{A1c} (57). The

importance of the effect of HbS found mainly in African-American populations on Hb_{A1c} assays is also apparent (58), although this is an effect which laboratories are aware of and therefore normally allow for. Factors that impact on red cell survival or those which regulate intracellular glucose concentrations - including glucose permeability across the red cell membrane, independent of extracellular glucose - have been shown also to contribute to the extent of hemoglobin glycation (23-25). The variability in the intracellular glucose relative to extracellular glycaemia significantly contributes to variation in HbA_{1c}. Other factors that influence non-enzymatic hemoglobin glycation include intracellular pH, 2,3-diphosphoglycerate concentration and glycolytic enzyme activity (36).

IX. Differential rates of intra-cellular glycation independent of glucose: fructosamine-3-kinase as a deglycating enzyme associated with the GGap

One possible explanation of the GGap that has been mooted is that of an enzyme-mediated intracellular deglycation process. We have recently adduced evidence of a potential role of the enzyme fructosamine-3-kinase (FN3K) enzyme in the GGap. FN3K has previously been shown to phosphorylate aldimine Amadori products of protein glycation at specific amino-groups in hemoglobin and other proteins, effectively deglycating the protein and restoring the free amino-group with the production of deoxyglucosone (59). FN3K is a predominantly intracellular enzyme expressed highly in erythrocytes (60). Single nucleotide polymorphisms (SNPs) in the FN3K gene have been shown to be associated variously with HbA_{1c} levels and circulating soluble receptors for AGE (sRAGE) (61, 62) and genome-wide association studies (GWAS) have found the *fn3k* gene to be one of the top hits for association with HbA1c (63, 64) this genetic variant being neither associated with glycemic traits nor erythrocytic indices.

We studied erythrocyte FN3K concentrations and enzyme activity in a sub-set of our diabetes patient population, dichotomised for a large positive or negative GGap (65). We showed that FN3K protein was significantly higher and, strikingly, that FN3K enzyme activity was 3-fold greater at any given FN3K protein level in the erythrocytes of the negative compared with positive GGap groups. This was associated with significantly lower advanced glycation end product (AGE) levels, lower pro-inflammatory adipokines (Leptin/Adiponectin ratio) and much lower pro-thrombotic PAI-1 levels in the negative GGap cohort, thus suggesting a possible role of FN3K as a deglycating enzyme in diabetes complications, potentially reducing some of the AGE involved in the pathogenesis of diabetes complications (65).

An objection to the potential role of FN3K in the GGap arises from its higher rate of activity in respect of fructosyl-lysines generated by glycation of side-chain amino-groups in proteins, coupled to its reportedly low activity on N-terminal amino-groups such as that of the N-terminal valine on Hb beta-chains (66). Thus the specificity of FN3K to N-ε-fructosyl-lysine (FruLys) compared with "N-terminal" N-α-fructosyl amino acids reportedly ranges from 100 times to 10 times lower affinity (67). This argument may be countered by considering the long time period over which FN3K may be able to act within the erythrocyte and since a lower affinity simply suggests a slower, but not zero-rate reaction (especially if the difference is only 10-fold) a significant degree of deglycation at the N-terminal valine may still occur. It must also be considered that published affinity values for FruLys comprise the free amino acid and the protein-bound or histone-bound FruLys, whereas for N-α–bound Amadori products only the free amino acids have been examined (59, 60). Indeed there is preliminary evidence of significantly measurable activity on N-α–bound Amadori products such as fructosyl-valine (Hellwig, personal communication). The fact that our studies have

demonstrated such a marked difference in FN3K activity in relation to GGap also supports the contention that it has a significant role in the GGap (65).

X. Clinical implications of GGap and HGI

The disagreement between HbA_{1c} and other measures of glycaemia including fructosamine or MBG, as calculated by GGap or HGI respectively, can be substantial in magnitude and is consistent over time (3-6, 27). Thus utilising HbA_{1c}, the current gold standard for assessment of glycemic control in diabetes, alone could potentially under or overestimate the prevailing glycemia, and leading to error in clinical assessment and management. Moreover use of the derived estimated average glucose (eAG) is more likely to result in overlooking the limitations of the HbA_{1c} measurement from which it is calculated (68). Individuals with a high GGap or high HGI, wherein the HbA_{1c} is higher than indicated by the serum fructosamine or MBG respectively, may receive an up-titration of their glycaemia treatment that may put them at undue risk of hypoglycemia if the GGap/ HGI is not taken into account (and several studies confirm that this does happen in practice). On the other hand in the case of those with a negative GGap / low HGI, wherein the HbA_{1c} is lower than the prevailing glycaemia, clinicians may be falsely reassured by HbA_{1c}, resulting in no appropriate therapy intensification to improve glycemia, putting such individuals at risk of diabetes-related complications. The HGI subgroup analysis in the ACCORD Trial suggested that the incidence of hypoglycemia was progressively higher in the low, moderate, and high HGI subgroups in both intensive (14.5, 16.8, and 18.8%, respectively) and standard (3.7, 4.5, and 7.5%, respectively) glycemia treatment cohorts (44).

HbA1c arguably still has value as a risk marker in diabetes risk stratification, prediction, diagnosis and management for many patients where clinical errors may be small however the

danger of not questioning its validity in the subset of individuals where there is potential for large errors is apparent from the discussion in the preceding paragraph. Setting aside considerations of its calculation, its impact on vascular risk and mortality and its possible underlying aetiology, the key purpose of this review is to raise awareness of the potential of HbA_{1c} inaccuracy for this subset of patients, reflected in the GGap (and HGI), to result in significant error in the assessment of glycemic control. In order to assess the possible scale of this error (and thus the probable size of the patient subset) we extended and re-analysed the data previously published (3) and we have recently reported the findings of this re-analysis (69). These findings are highlighted in Figure 1 which shows our total accrued data on 31,119 simultaneously measured HbA1c and fructosamine estimations undertaken in our single center over 10 years. It is apparent from this figure that there is wide scatter around the line of unity which, in absolute terms, was > than 1 unit of glycated HbA_{1c}% or 7.2 mmol/mol in 40% of estimations.

Figure 2 shows how this scatter results in differences in the categorisation of glycemic attainment between HbA_{1c} and fructosamine-derived $FHbA_{1c}$. Whilst many people with diabetes in our center would be accurately categorized for glycemic attainment based on HbA_{1c}, a large proportion would be not be. This is demonstrated in Figure 3 which depicts the magnitude of the variance in that categorisation, with only 46% showing concordance and 15% of patients having, in our opinion, a large enough difference to impart certain risk by way of error in a clinician's judgement, with consequent potential for inappropriate therapy and management.

It may thereby be, as reported by ACCORD (42), that those in lower attained HbA_{1c} brackets with a higher HGI, who were perhaps exposed to therapy intensification inappropriately,

came to harm. A recent review by Campbell *et al.* (24) suggests that the GGap is unlikely to cause such errors since "*in the main initiation and alterations of diabetic therapies are almost never made based on an isolated HbA$_{1c}$, particularly at levels close to the diagnostic threshold*", however as we have seen, because of the individually consistent nature of the GGap, multiple measurements of HbA_{1c} are likely to yield similar conclusions and furthermore as we have shown in the preceding paragraph common variations as small as 1% in HbA_{1c} from mean glycemia-predicted HbA_{1c} can result in significant clinical errors. This is further supported by Cohen *et al.* (2) quoting a recent study by Rhee *et al.* (70) who found in a "*VA population that those whose HbA1c is highest relative to blood glucose* (ie equivalent to a positive GGap) *had a 56% higher frequency of ER visits for hypoglycemia than those whose HbA1c is either proportionate or lowest for blood glucose* (ie a neutral or negative GGap)" (non-italicized insertions are ours).

Understanding the association of GGap and HGI with key diabetes-related microvascular complications, as suggested in various studies, in a pattern consistent with key pathophysiological mechanism namely glycation of proteins, would help to risk-stratify such individuals for targeted risk reduction therapies, and also help in future to develop pharmacological interventions aimed towards risk reduction. The observations of significantly different outcomes in the different HGI subgroups in the ACCORD trial in response to intensive treatment strongly supports the need for more personalized diabetes management and suggests that HGI could be used to help individualize treatment goals.

Given the standardization of HbA_{1c} assays, ease of its estimation and practicality of its use in treatment modification (based on available evidence for association with complications and improved outcomes with HbA_{1c} reduction), HbA_{1c} is now adopted internationally for

diagnosis of diabetes. However the magnitude of GGap/HGI means that this reliance may have marked impact in a significant minority of cases. HbA_{1c} alone may not always be reliable for diagnostic purposes, with studies showing a low sensitivity of HbA_{1c} for diagnosis, leading to substantial number of missed diagnoses and to error in classification of diabetes status, in the absence of concurrent use of available glucose criteria for diabetes diagnosis. Rodriguez-Segade *et al.*, in a study on patients with previously undiagnosed diabetes, confirmed that the differences between HbA_{1C}-based and fasting plasma glucose/OGTT-based diagnoses are largely due to the influence of the GGap calculated using simultaneously measured serum fructosamine (71). As previously mentioned, the increasing availability of CGM in some areas has the potential to provide an alternative to Hb_{A1c} in assessing glycemic control and Beck, Bergenstal and colleagues have suggested the use of a factor derived from CGM which they term the Glucose Management Indicator (GMI) (72, 73). However, as pointed out above (see the end of section IV) CGM is still not available in many constituencies and in others is limited (for funding and other reasons) to a small fraction of diabetes patients (for example in the UK, where strict NHS guidelines restrict provision to patients with type 1 diabetes having poor control *ie* substantially less than 5% of diabetes patients); furthermore, as we describe in the previous paragraph, determination of GGap or HGI (unlike GMI) has the additional benefit of providing a prognostic indicator or risk of diabetic complications.

XI. CONCLUSIONS

In summary, GGap/ HGI can be sufficient in magnitude to cause an error in the judgment of glycemia attainment. Hence the incorporation of GGap/ HGI during assessment of glycemic control would help to ascertain how far HbA_{1c} diverges from alternative estimates of glycaemia, to avoid misinterpretation of glycemic control and to avoid inappropriate

therapeutic management. Understanding the consistency of GGap, its association with a phenotype in diabetes, microvascular complications, macrovascular disease and possibly mortality, and its possible mechanistic association with FN3K enzyme activity will also contribute towards directing further research into emerging therapeutic interventions to lessen diabetes related complications.

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Table 1: Studies investigating the association of diabetic complications with the glycation gap (GG) and the hemoglobin glycation index (HGI)

Study (Reference no.)	Patient population	Results
GGap (GG) and complications		
Cohen *et al*. 2003 (5)	40 patients with type 1 diabetes of >15 years duration	GG increase by 1% was associated with 2.9 fold greater frequency of adverse nephropathy stage (P=0.0014)
Rodriguez-Segade *et al*. 2011 (42)	2,314 patients with type 2 diabetes	High GG associated with progression of nephropathy in type 2 diabetes HR (high vs. low GG)=2.52(P<0.001) and HR (medium vs. low GG)=1.61(P=0.001)
Nayak *et al*. 2013 (41)	3,182 patients with type 1 and type 2 diabetes	Positive GG is associated with retinopathy (OR 1.24 [95% CI 1.01–1.52, P=0.039], nephropathy (1.55 [1.23– 1.95, P=0.008]) and macrovascular disease (1.91[1.18–3.09]) GG had a "U"-shaped quadratic relationship with mortality: negative G-gap (1.96 [1.50-2.55], P<0.001) and positive G-gap (2.02 [1.57–2.60], P<0.001) being associated with a significantly higher mortality.
Cosson *et al*. 2013 (48)	925 patients with type 2 diabetes	High GG (third tertile of GG) was associated with macroproteinuria (1.6 [1.2–2.1]; $P < 0.01$) independent of HbA_{1c}
Cid Alvarez *et al*. 2012 (45)	1,137 patients admitted with Acute Coronary syndrome	GG was associated with a significantly higher risk of all cause mortality in both diabetic [HR IC 95%: 1.31 (1.14-1.50), P=0.000] and non-diabetic patients [HR IC 95%: 1.30 (1.04-1.64), P=0.018].
HGI and complications		
McCarter *et al*. 2004 (41)	1,441 DCCT participants with type 1 diabetes	High HGI group had greater risk of retinopathy (3-fold) and nephropathy (6-fold) compared with low HGI group
Lachin *et al*. 2007 (34)	1,441 DCCT participants with type 1 diabetes	The effect of the HGI on microvascular complications in DCCT cohort is wholly explained by the associated level of HbA_{1c}
Hempe *et al*. 2015 (44)	10,251 patients with type 2 diabetes (ACCORD cohort)	Total mortality in intensively treated patients was higher in high HGI subgroup (HR 1.41 [95% CI 1.10– 1.80]) High HGI was associated with a greater risk for hypoglycemia in the standard and intensive treatment groups.
van Steen *et al*. 2018 (46)	11,083 patients with type 2 diabetes (ADVANCE trial cohort)	High HGI is a predictor of micro- and macro vascular complications and mortality but no better than HbA_{1c} High HGI associated with lower risk for mortality when on intensive treatment
Rhee *et al*. 2017 (49)	2,052 non-diabetic individuals	High HGI associated with higher risk for incident Coronary artery calcifications independent of HbA_{1c}
Fiorentino *et al*. 2017 (50)	1,120 Caucasians without diabetes	High HGI associated with 2 fold increased risk of hepatic steatosis in non-diabetics
Cheng *et al*. 2017 (51)	423 individuals with type 2 diabetes (Taiwan)	HGI correlated with the extent of CHD in individuals with Type 2 diabetes
Marini *et al*. 2017 (52)	2,055 white non-diabetic adults age >=18 years	HGI is a predictor of Carotid intima-media thickness. Individuals with high HGI had 2.7-fold increased risk of vascular atherosclerosis (OR 2.72 (1.01-7.37)) as compared to individuals with low HGI
Pan *et al*. 2017 (47)	976 diabetic patients with ischaemic stroke (China)	Both high and low HGI were linked to poor outcome in acute ischaemic stroke [U-shaped association with OR (95% CI) for low vs. moderate HGI group = 1.64(1.13-2.38), P=0.01; and high vs. moderate HGI = 1.54(1.06-2.24), P= 0.02]
Ahn *et al*. 2017 (53)	248 treatment-naïve subjects with pre-diabetes or diabetes	Highest HGI tertile was independently associated with composite CVD [OR (95% CI): 2.81 (1.59-4.98)], and individual CAD [2.30 (1.12-4.73)], stroke [3.40 (1.50-7.73)], and PAD [6.37 (1.18-34.33)] after adjustment for other CVD risk factors including HbA1c levels.

FIGURE LEGENDS:

Figure 1: Clinical grid showing variation in categorization of actual HbA_{1c} and estimated fructosamine-derived HbA1c in a single diabetes center over 10 years

The figure shows 31,119 simultaneously measured HbA_{1c} and Fructosamine estimations. F_HbA1c is the derived HbA_{1c} estimated from Fructosamine. The grid shows the levels at which HbA_{1c} and F_HbA_{1c} can be arbitrarily categorised as Excellent (≤ 7% (53.0 mmol/mol), E), Good (7-8% (53.1-63.9 mmol/mol), G), Acceptable (8-9% (64.0-74.9 mmol/mol), A), Poor (9-10% (75.0-85.8 mmol/mol), P) or Very Poor (>10% (85.8 mmol/mol), VP). Glycated hemoglobin levels are depicted in DCCT units for simplicity. All values are shown with the degree of scatter around the line of unity whilst horizontal and vertical lines represent the defined categories.

Figure 2: Differences in clinical categorization using actual HbA_{1c} and estimated fructosamine-derived HbA_{1c}

The figure compares the categories of HbA_{1c} and F_HbA_{1c} based on the data depicted in figure 1. The glycemic control categories on the X-axis (E, excellent; G, good; A, acceptable; P, poor; VP, very poor) are those defined using actual HbA_{1c} and the colors show the categories that would be derived for the same measurements if based on fructosamine-derived F_HbA_{1c}. (Red - excellent, Green – good, Blue – acceptable, Orange – poor, Yellow – very poor).

Figure 3: Magnitude of variation between glycemic control categories arising from the glycation gap

The figure shows the magnitude of the variance between the categories defined in figure 2. This sums all variations which are in agreement, those which are 1 block different, or those which are 2 blocks or more different. Less than half (46%) of the paired measurements give categorization results which agree when comparing HbA1c and F_HbA1c. 54% disagree by at least 1 block and of these 15% disagree by 2 blocks of category or more potentially leading to serious clinical misjudgements.

Figure 1:

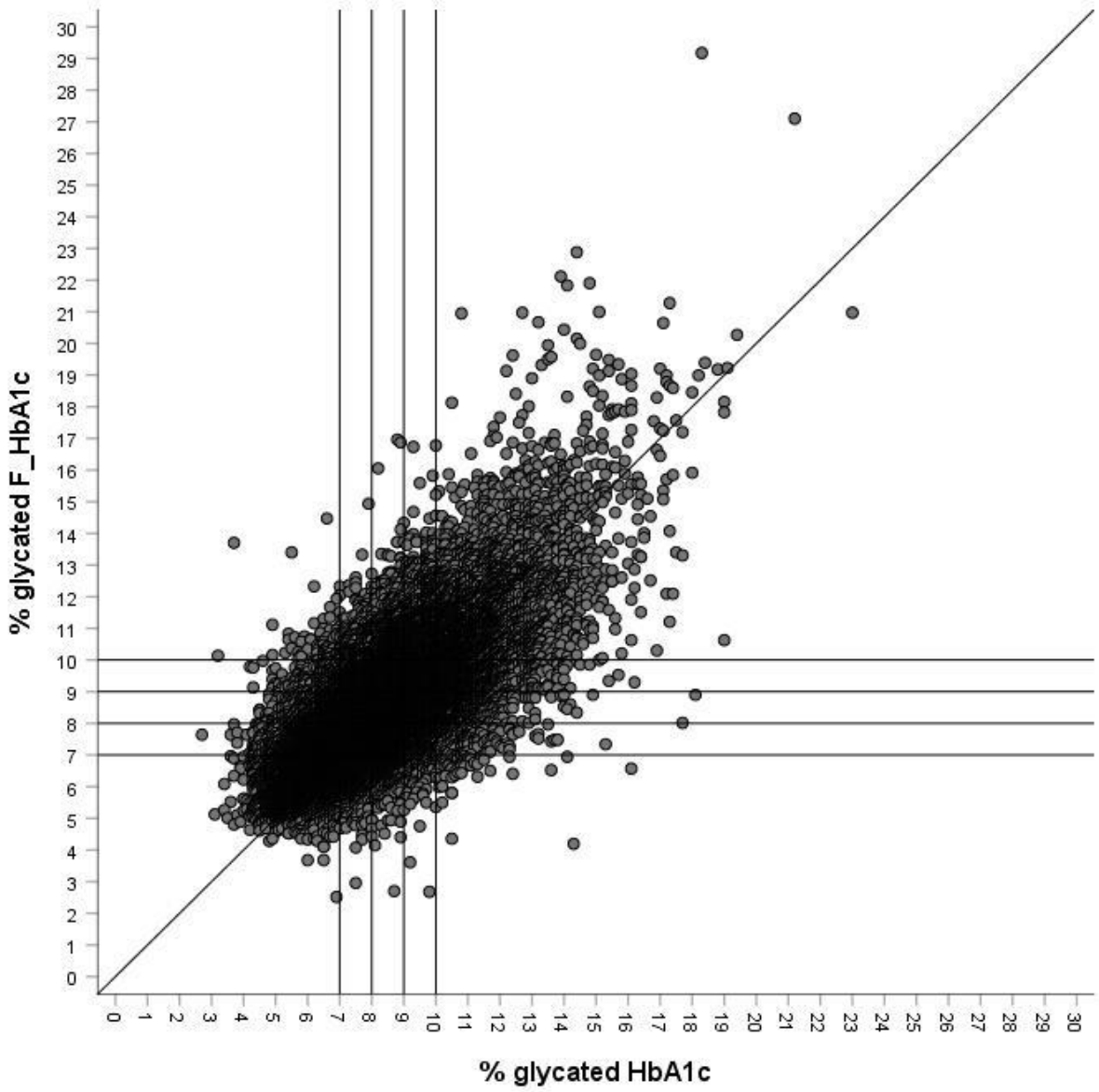

% glycated F_HbA1c
% glycated HbA1c
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 2:

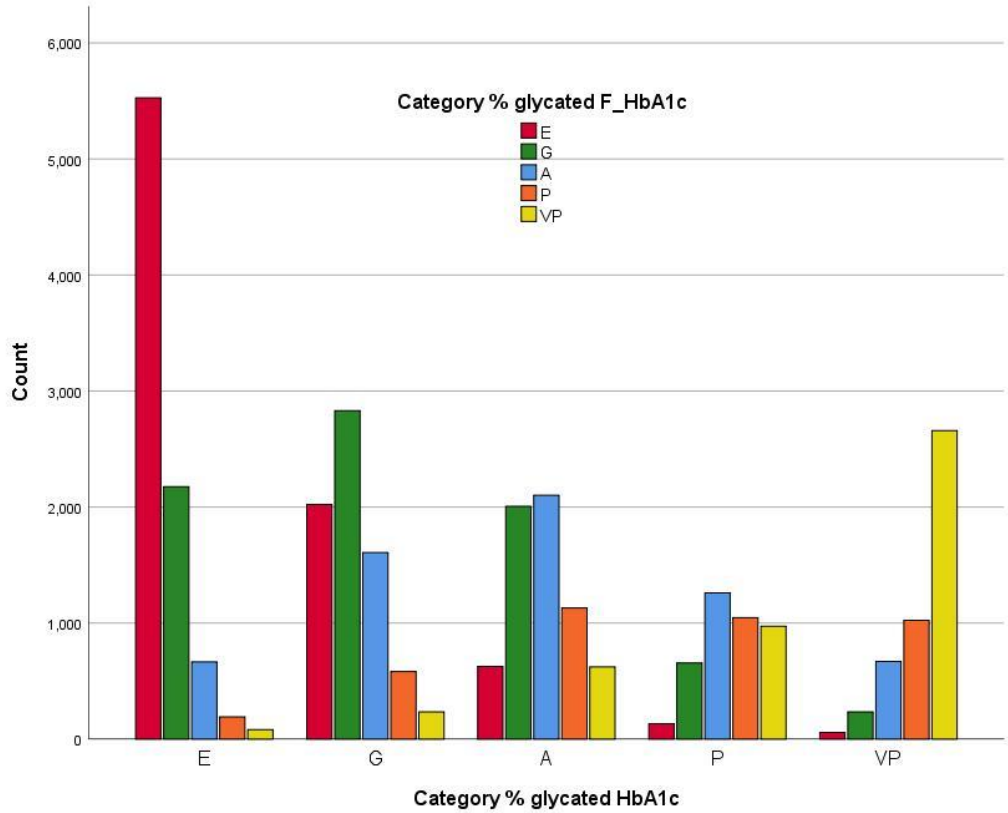
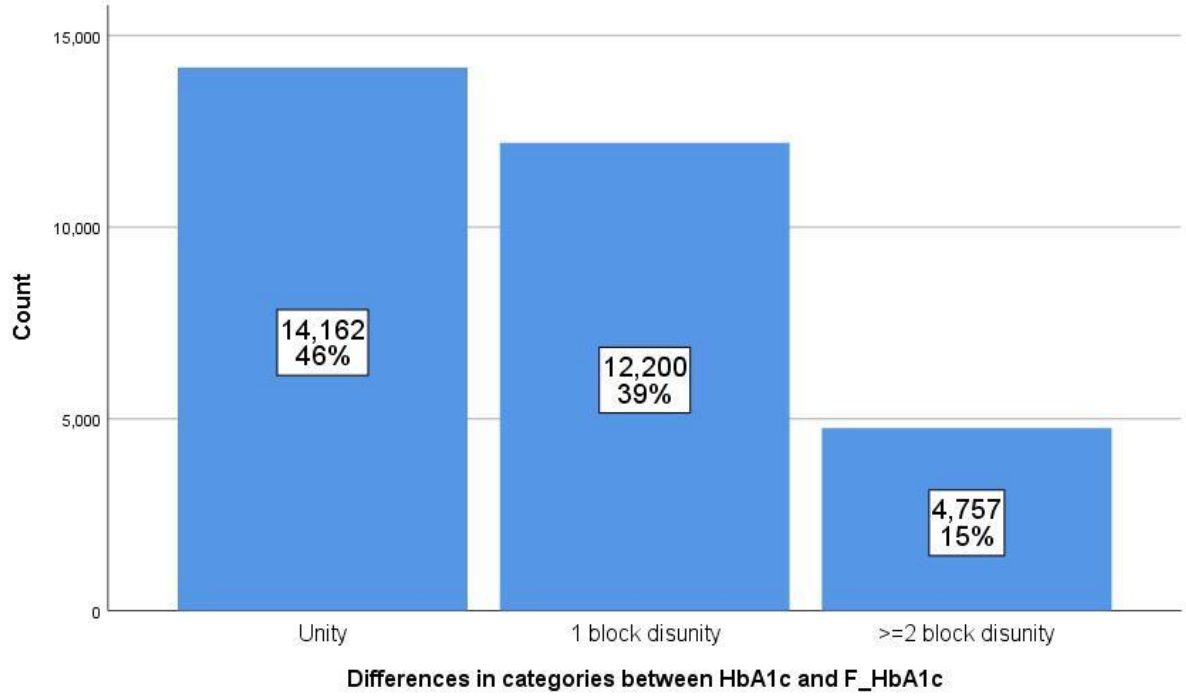

Category % glycated F_HbA1c
E
G
A
P
VP
Count
6,000
5,000
4,000
3,000
2,000
1,000
0
E
G
A
P
VP
Category % glycated HbA1c

Figure 3:


15,000
10,000
5,000
0
Count
14,162
46%
12,200
39%
4,757
15%
Unity
1 block disunity
>=2 block disunity
Differences in categories between HbA1c and F_HbA1c